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Immune-Mediated Thrombocytopenia in Horses Infected with Equine Infectious Anemia Virus

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An adult horse infected with a virulent, cell culture-adapted strain of equine infectious anemia virus (EIAV) developed cyclical thrombocytopenia in which the nadir of platelet counts coincided with peak febrile responses. In order to investigate the mechanism of thrombocytopenia during acute febrile episodes, four adult horses were experimentally infected with the wild-type Wyoming strain of EIAV. Platelet counts decreased from baseline as rectal temperature increased. Serum reverse transcriptase activity increased above background levels in all horses, coincident with increase in rectal temperature. All horses developed an EIAV-specific immune response detectable by Western immunoblot by postinfection day 10. Increases in platelet-associated immunoglobulins G and M were detectable by direct fluorescent-antibody test and flow cytometric assay. Viral replication in bone marrow megakaryocytes was not detectable by in situ hybridization. Results suggest an immune-mediated mechanism of thrombocytopenia in horses infected with EIAV. Despite an inability to identify virion particles in association with platelet-bound antibody, the cyclical nature of the thrombocytopenia and the occurrence of a marked cell-free viremia concomitant with fever and thrombocytopenia suggest immune complex deposition on platelets. We propose that clearance of virus and antibody-coated platelets from the peripheral circulation by hepatic Kupffer cells and splenic macrophages may target infectious virus particles, in the form of immune complexes, to host cells most permissive for in vivo viral replication.

Equine infectious anemia virus (EIAV) is a lentivirus that shares considerable homology with human immunodeficiency virus types 1 and 2 (HIV-1 and HIV-2) (8, 12, 13, 26, 40). Unlike infection with most other lentiviruses, infection of horses with EIAV frequently results in acute febrile illness within 1 to 2 weeks and is followed by a syndrome of recrudescing fever, weight loss, and anemia (15). Each febrile episode is associated with emergence of a new antigenic variant of the virus (27, 30, 35) and a high-titer, cell-free viremia (15). A consistent hematologic finding in febrile animals is thrombocytopenia (15), the cause of which has never been investigated. Viral-induced thrombocytopenia may result from immune-mediated clearance, nonimmune clearance, or marrow suppression. Thrombocytopenia has been documented in some humans infected with HIV-1 and associated with immune complex deposition on platelets (17), anti-platelet antibody (42), and active infection of megakaryocytes in the bone marrow (49). EIAV has been shown to exist in sera of infected horses as an infectious virus-antibody immune complex (22), and immune mechanisms appear to play an important role in many aspects of pathogenesis. The anemia in horses persistently infected with EIAV has been attributed to both immune-mediated clearance of complement-coated erythrocytes (23, 25, 31, 37–39) and generalized bone marrow suppression (24, 32). Immune complex glomerulonephritis is seen in many chronically infected horses (5). We hypothesized that the thrombocytopenia of acute EIAV infection is, at least in part, immune mediated. To test this hypothesis, we have examined platelets from acutely infected horses for changes in quantity of surface immunoglobulins G and M (IgG and IgM) and for platelet-associated virus. We have also examined bone marrow for the presence of viral replication in megakaryocytes or megakaryocyte depletion.

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MATERIALS AND METHODS

Virus. The wild-type Wyoming virus strain 158 was obtained from L. O. Mott (18) and propagated in horses. Serum was obtained from an infected horse during the peak febrile response, and the titer of this stock (hereafter referred to as wild-type Wyoming virus strain 158 serum) was determined to be approximately 10^6 infectious doses per ml (as determined by horse inoculation assay). The Malmquist strain of EIAV was derived from the wild-type Wyoming strain by adaptation of the wild-type virus in equine dermis fibroblast cells (29).

Platelet isolation. Heparinized blood was collected from the jugular vein of all horses prior to infection and on postinfection days (PIDs) 2, 4, 7, 9, 10, and 11 (also on PIDs 14 and 16 from horses E4313 and E4316). Samples were collected from an uninfected control horse on each day. Erythrocytes were allowed to settle, and plasma was harvested and centrifuged at 100 × *g* for 10 min to remove leukocytes. Aliquots of platelet-rich plasma were centrifuged at 13,000 × *g* for 3 min in a microcentrifuge to pellet platelets. Platelets were resuspended and washed twice in 9:1 phosphate-buffered saline (PBS) and 0.13 M Na citrate. Washed platelets were counted by using an automated cell counter (Coulter).

Direct fluorescent-antibody test. Platelets were centrifuged onto glass slides at a density of approximately 10^7 platelets per slide. Slides were allowed to air dry for 5 min and were used immediately. (Some slides were fixed in 4% paraformaldehyde, dehydrated through graded ethanol, and stored at

−70°C until used for in situ hybridization, as described below.) Slides were treated for 10 min with 10% normal goat serum in PBS to block nonspecific binding and washed twice for 3 min each with PBS. Slides were then incubated with fluorescein isothiocyanate (FITC)-conjugated antibody or PBS for 10 min at 37°C. FITC-conjugated goat anti-equine IgG (gamma chain specific) and IgM (mu chain specific) (Kirkegaard & Perry Laboratories) were used to detect platelet-associated surface IgG and IgM, respectively. FITC-conjugated goat anti-mouse IgG and IgM (Kirkegaard & Perry Laboratories) were absorbed over equine lymphoid tissue and used as negative controls. After incubation with labeled antibody, slides were washed twice for 3 min each with PBS, counterstained for 5 min in 0.125% Evans blue, rinsed briefly in PBS, and examined by fluorescence microscopy. Pictures of representative microscopic fields were examined by three blinded observers to assess increases in platelet fluorescence. The day at which increased fluorescence was apparent to all observers was designated as the first day of increased platelet-associated Ig (see Fig. 2).

Flow cytometric analysis. Aliquots of 10^7 platelets were incubated with 100 μl of FITC-conjugated antibody (as above) for 1 h at 4°C. Platelets were washed once in 9:1 PBS and 0.13 M Na citrate. The number and intensity of fluorescing platelets were determined by flow cytometric analysis with a Becton Dickinson FACScan. Washed platelets were also incubated with saline and FITC-conjugated goat anti-mouse IgG (Kirkegaard & Perry Laboratories) as negative controls. On each day, samples from an acutely infected horse were analyzed alongside similar platelet preparations from an uninfected control horse.

Western immunoblots. The Malmquist strain of EIAV was harvested by ultracentrifugation of the supernatant fluids from persistently infected equine dermis cell cultures and concentrated by banding on sucrose gradients. EIAV proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (19) on 10% gels. The proteins were transferred to Immobilon-P membranes (Millipore Corp.) and reacted with pre- or postinfection sera and then with phosphatase-labeled goat anti-horse IgG (Kirkegaard & Perry Laboratories). Reactions were developed with a model SK 5200 alkaline phosphatase substrate kit II (Vector Laboratories, Inc.). Western immunoblots of platelets were performed similarly. Platelet lysates were electrophoresed as above, transferred to membranes, and reacted with a polyclonal equine anti-EIAV serum.

RT assay. Serum reverse transcriptase (RT) activity in all horses was measured prior to infection and on selected PIDs. One milliliter of cell-free serum was centrifuged at 13,000 × *g* at room temperature for 1 h. The precipitant was resuspended in 10 μl of suspension buffer (50 mM Tris-HCl [pH 7.5], 1 mM dithiothreitol, 20% glycerol, 0.25 M KCl, 0.25% Triton X-100). Samples were frozen on dry ice and thawed at 37°C for a total of three freeze-thaw cycles. Samples were then mixed with 20 μl of RT cocktail consisting of 50 mM Tris-HCl (pH 7.9), 50 mM KCl, 5 mM $MgCl_2$, 5 mM dithiothreitol, 0.05% Nonidet P-40, 0.33 A_{260} unit of poly(rA)-poly(dT)$_{12-18}$ per ml, and 0.5 μM [^{3}H]TTP (62 Ci/mmol). Reaction mixtures were incubated for 90 min at 37°C, and 25 μl of each sample was spotted on Whatman DE81 filters. Filters were washed 3 times in 0.35 M Na_2HPO_4, rinsed briefly in distilled H_2O, and washed once in 95% ethanol. Filters were air dried and counted in Bio-Safe II (Research Products International Corp.) liquid scintillation-counting cocktail with an automated beta counter (Pharmacia LKB). For two horses (E390 and E4316), RT activity was also determined after viral purification from 10-ml aliquots of serum. Serum was layered on 3 ml of 20% sucrose in TNE (25 mM Tris-HCl [pH 7.4], 0.15 M NaCl, 2 mM EDTA) and centrifuged at 100,000 × *g* at 4°C for 1 h. The pellet was resuspended in 100 μl of PBS, and a 10-μl aliquot of the solution was assayed for RT activity as described above. Better sensitivity and lower backgrounds were seen in results from samples pelleted through a sucrose cushion compared with those from serum obtained at the same time but pelleted directly.

Bone marrow analysis. Bone marrow samples were obtained from one rib from each of two horses (E390 and E516) at necropsy. Samples for in situ hybridization were fixed overnight in 4% paraformaldehyde, decalcified overnight in RDO solution (Dupaige Kinetic Laboratories), paraffin embedded, and cut in 3-μm sections to be mounted on poly-L-lysine-coated slides. Other marrow sections were processed with formaldehyde fixation and hematoxylin and eosin staining for routine histopathologic examination. Bone marrow aspirates were obtained from the sternums of all horses immediately after sacrifice. Marrow smears were made on glass slides and stained with a Giemsa-type stain (DifQuik) for routine cytologic examination or fixed with paraformaldehyde and dehydrated for in situ hybridization.

Probe preparation. An 825-bp *Kpn*I-*Bgl*II fragment from the *gag* gene of a cell culture-adapted, proviral clone of EIAV was cloned into the *Kpn*I-*Bam*HI site of the transcription vector pGEM-4Z (Promega). The plasmid was purified and linearized with the restriction enzyme *Apa*I. Independent in vitro transcription reactions were performed with SP6 and T7 polymerases (Promega) as described previously (3), by using α-^{35}S-UTP. The percent incorporation of α-^{35}S-UTP in the final probe was typically about 70 to 80% (specific activity, approximately 10^9 cpm/μg). The SP6 polymerase reaction resulted in an RNA probe complementary to viral message and 280 bases in length. Transcription with the T7 polymerase produced a message sense probe 545 bases in length. Probe activity was quantitated by counting of 1-μl samples in scintillation fluid with an automated beta counter (Pharmacia LKB).

In situ hybridization. In situ hybridization was performed as previously described (3, 14), with the following modifications. Briefly, bone marrow samples were deparaffinized with xylenes, rehydrated through graded ethanol, and denatured sequentially with 0.2 N HCl, 2× SSC at 70°C (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 1 μg of proteinase K per ml in 20 mM Tris-HCl and 2 mM $CaCl_2$. Cells were refixed briefly in fresh 4% paraformaldehyde and rinsed with PBS. Nonspecific sulfur binding was blocked with 0.01 M *N*-ethylmaleimide and 0.01 M iodoacetamide in PBS. Nonspecific binding to the glass slides was blocked with 0.25% acetic anhydride in 0.1 M triethanolamine buffer. Slides were dehydrated again through graded ethanol prior to hybridization. Each slide was hybridized with 50,000 cpm of appropriate probe in 5 μl of hybridization mix under a siliconized coverslip. Slides were immersed overnight in mineral oil at 45°C. Slides were hybridized in duplicate with SP6 and T7 probes. Control samples included equine dermal cells infected with a cell-adapted strain of EIAV, uninfected equine dermal cells or Vero cells, and liver sections from the acutely infected horse. After hybridization, slides were rinsed in chloroform twice to remove residual mineral oil, and coverslips were floated off in 2× SSC. Washing and RNase treatment to remove nonspecifically bound probe were performed as described previously (3). Slides were dehydrated through graded ethanol and dipped in Kodak

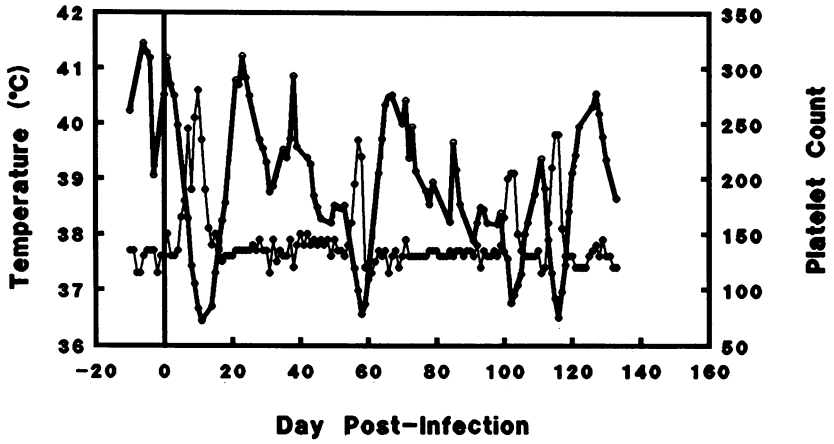

FIG. 1. Platelet count ($10^3/\mu l$) (open circles) and rectal temperature (closed circles) of horse E244 after infection with the cell culture-adapted Malmquist strain of EIAV that had been passaged through horses to reestablish virulence.

NTB-2 emulsion. They were stored in a light-tight slide box at 4°C for 4 to 6 days. After development with Kodak developer and fixer, slides were rinsed, dried, and routinely stained with hematoxylin and eosin. In situ hybridization of platelet cytospin slides was performed as above, but without xylene deparaffinization and without proteinase K digestion.

RESULTS

Response to infection. One horse was infected with the cell-adapted Malmquist strain of EIAV that had been previously passaged through horses to reestablish virulence (29). Rectal temperature and platelet counts were monitored through repeated febrile episodes to document kinetics of platelet decline and rebound (Fig. 1). This experiment demonstrated that the thrombocytopenia coincided with febrile episodes and cell-free viremia (see below). The rapid rebound of platelets following each febrile episode suggested that an immune-mediated clearance or destruction of platelets rather than a bone marrow suppression of platelet production was occurring. To investigate this further, we sought to test the hypothesis that an immune-mediated clearance was at least in part responsible for the thrombocytopenia.

A total of four horses were infected intravenously with 1 ml of wild-type Wyoming virus strain 158 serum. All horses developed a rectal temperature of at least 40°C by PID 11, with a concomitant decrease in platelet counts to <50,000/μl by PID 14 (Fig. 2). (Horse E516, whose platelet count declined to 95,000/μl by PID 10, was sacrificed at that time.) Platelet count changed inversely with increasing rectal temperature. Other consistent hematologic abnormalities included declining erythrocyte numbers and slight leukopenia (data not shown). All horses became depressed and exhibited diminished appetites during the peak febrile response. None of the horses showed signs of petechial hemorrhages of mucosal membranes or increased bleeding following venipuncture, even when platelet counts were extremely low (<30,000/μl). One exception was horse E4313, which developed a large hematoma after jugular venipuncture 1 h prior to sacrifice on PID 18. Horse E4313 also exhibited severe diarrhea and grossly lipemic plasma on the day of euthanasia. A complete necropsy of horses E390 and E516 revealed gross pathological findings of splenomegaly, hepatomegaly, and enlargement of multiple internal lymph nodes. Microscopically, erythrophagocytosis was seen in the spleen and bone marrow. These findings are consistent with previous descriptions of EIAV-induced disease (15).

The earliest PID at which increases in serum RT are detectable over background levels is shown in Fig. 2 (down arrows). All horses developed an antibody response to the p26 core protein of EIAV, as detected by Western immunoblot, by PID 10 (data not shown).

Direct immunofluorescence of platelets. To identify increased platelet-bound antibody, platelets from infected horses were analyzed by direct immunofluorescence by using FITC-conjugated anti-equine IgG and IgM. Platelet cytospin samples from all horses showed a small number of highly fluorescent platelets prior to infection. Control horses maintained this background fluorescence throughout the experimental period. This is not surprising, since platelets have F_c receptors on their surface and can bind antibody. However, infected horses developed markedly increased numbers of highly fluorescent platelets, with fluorescence peaking at the time peripheral blood platelet counts reached their nadir (Fig. 3). Fluorescence of platelets from these horses was increased as early as PID 4 (Fig. 2, up arrows) and generally increased from that day until sacrifice. Although increases in both platelet-associated IgG and IgM were seen for all horses, increases in IgM were more marked (Fig. 3).

Flow cytometric analysis of platelets. To better quantitate the increase in platelet-associated antibody following infection, platelets from infected and uninfected horses were reacted with FITC-conjugated antibodies specific for IgG and IgM as above and then analyzed by flow cytometry (Fig. 4 and 5). A mean fluorescence index for both IgG and IgM was determined for each horse by dividing the mean fluorescence intensity of platelets stained with appropriate FITC-conjugated antibody by the mean fluorescence intensity of unstained platelets. The mean IgG and IgM fluorescence indices for platelets from 14 uninfected horses were determined in order to calculate the upper limit of normal (mean + 2 standard deviations) for IgG and IgM, as indicated in Fig. 4. When platelets from three acutely infected horses (E516, E4313, and E4316) were analyzed, two patterns of change in fluorescence were observed. In horses E4313 and E4316, the fluorescence index of the main population of platelets increased during the experimental period

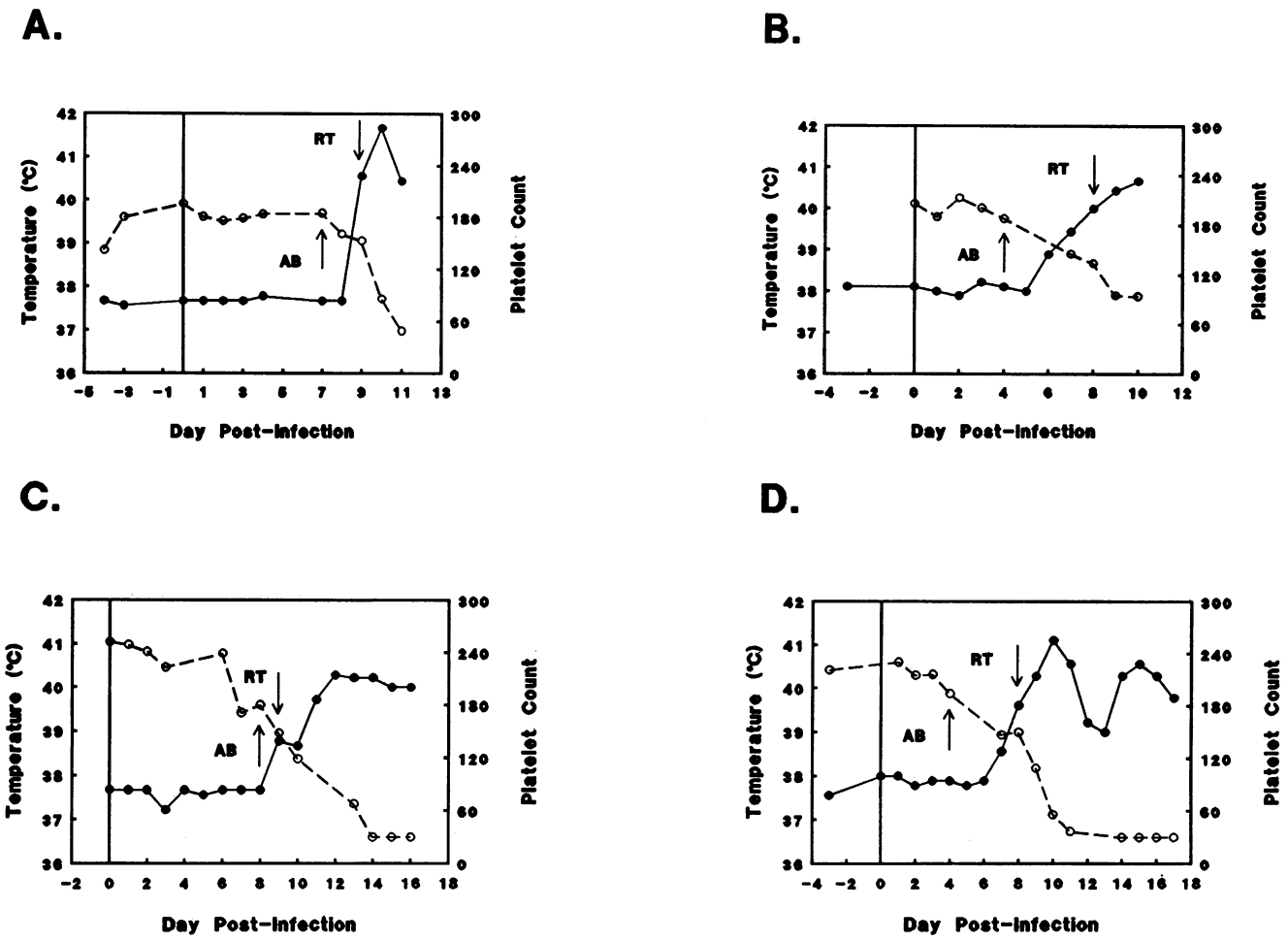

FIG. 2. Platelet count ($10^3/\mu l$) (open circles) and rectal temperature (closed circles) of horses E390 (A), E516 (B), E4316 (C), and E4313 (D) after infection with 1 ml of wild-type Wyoming virus strain 158 serum. Up arrows (labeled AB) and down arrows (labeled RT) indicate the earliest PID at which increased platelet-associated antibody (IgG and/or IgM) and increased serum RT activity, respectively, were detected. On the day of sacrifice, direct pelleting of serum resulted in the following peak RT activities (test value minus background counts per minute per 10 μl): 267 cpm for horse E390; 6,164 cpm for horse E516; 665 cpm for horse E4316; and 14,718 cpm for horse E4313. When serum was pelleted through a sucrose cushion, RT activity peaked at 4,500 cpm for horse E390 and at 29,000 cpm for horse E4316.

(Fig. 4) and the fluorescence index exceeded the upper limit of normal at the nadir of platelet counts (Fig. 2). Increases above normal were most marked for platelet-associated IgM (Fig. 4B). On PID 15, platelets from horse E4313 changed fluorescence pattern and developed a subpopulation of platelets with extremely marked fluorescence, while the major population of platelets showed a decrease in the fluorescence index (data not shown). In horse E516, the fluorescence index of the major low-fluorescence subpopulation of platelets never changed (data not shown), but a similar subpopulation of platelets with extremely marked fluorescence (shown on PID 9 in Fig. 5) increased in percentage throughout the experimental period (Fig. 5).

Platelet-associated virus. Platelet-associated IgG and IgM may occur as free antibody or in the form of virus-antibody immune complexes. Attempts to assess platelet-associated viral antigen by indirect immunofluorescence by using a rabbit polyclonal anti-EIAV antiserum were unsuccessful (data not shown). In addition, in situ hybridization of platelets from infected horses was negative for viral RNA, and Western immunoblot of platelet lysates was unsuccessful in detecting viral proteins.

Bone marrow analysis. Because thrombocytopenia can result from, or be exacerbated by, bone marrow suppression, megakaryocytes from infected horses were evaluated. Prior to infection, bone marrow aspirates from horses showed normal numbers of megakaryocytes on routine examination (data not shown). At the time of necropsy, no horse had grossly decreased numbers of bone marrow megakaryocytes; a marked increase in the number of these cells could not be documented. Such an increase should eventually be evident if thrombocytopenia was due solely to increased peripheral clearance of antibody-coated platelets (see Discussion). In situ hybridization of bone marrow samples from two horses (E390 and E516) showed a very high background of silver grains because of nonspecific binding of radioactive probe to eosinophils, but megakaryocytes had relatively few associated silver grains (Fig. 6C). Subjective observation of over 100 megakaryocytes from each horse found no megakaryocytes with overlying silver grains of the density seen in infected-liver specimens (Fig. 6D). To quantitate this observation, silver grains overlying 20 megakaryocytes were counted on slides hybridized with both the T7 and the SP6 probes (data not shown). Megakaryo-

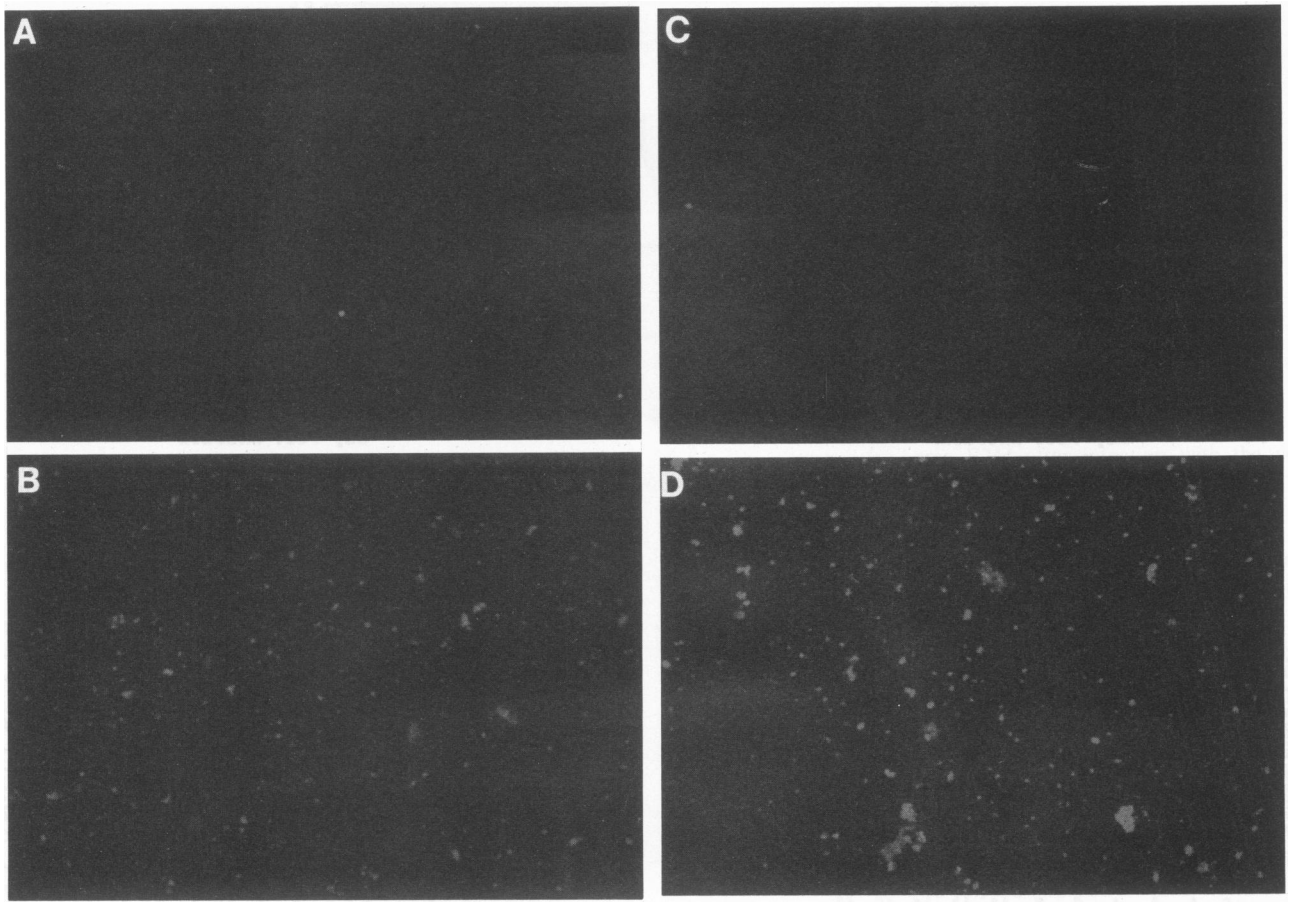

FIG. 3. Direct fluorescent-antibody test of platelets from horse E516. Washed platelets were centrifuged onto microscope slides and incubated with FITC-conjugated goat anti-equine IgG (A and B) or IgM (C and D). (A) Preinfection, IgG; (B) PID 9, IgG; (C) preinfection, IgM; (D) PID 9, IgM.

cytes from horse E390 had 7.0 ± 4.0 (mean ± standard deviation) and 5.3 ± 3.3 silver grains when hybridized with the SP6 probe and T7 probe, respectively. Megakaryocytes from horse E516 had 8.0 ± 3.1 and 7.0 ± 4.1 silver grains when hybridized with the SP6 probe and T7 probe, respectively. A Student's t test for comparison of sample means showed no significant difference ($P < 0.05$) in mean number of silver grains overlying megakaryocytes hybridized with the T7 or SP6 probe for either horse.

DISCUSSION

The kinetics of thrombocytopenia in EIAV-infected horses resembles that seen in other immune-mediated thrombocytopenias, especially in the rapid rebound in platelet counts seen with the resolution of fever (Fig. 1). Decreases in platelet count occur concomitant with increases in platelet-bound IgG and IgM and with increases in serum RT activity (Fig. 2). The nadir of platelet counts coincides with detection by Western immunoblot of virus-specific antibody free in serum. The presence of splenomegaly and hepatomegaly at necropsy is consistent with immune-mediated platelet destruction. Although a concomitant depression of megakaryocyte response and marrow platelet release cannot be ruled out on the basis of these findings, there was no subjective evidence of megakaryocyte depletion in the bone marrow. Additionally, by using the extremely sensitive technique of in situ hybridization, no evidence of active viral infection of megakaryocytes was found (Fig. 6). Therefore, the findings reported here are most consistent with a theory of immune complex deposition on platelets and subsequent destruction of those platelets by mononuclear phagocytes in the spleen and liver.

Direct fluorescent-antibody testing of washed platelets revealed a marked increase in platelet-associated IgM and IgG, first detectable as early as PID 4 (Fig. 2) and generally increasing until sacrifice (Fig. 4). This method of evaluation of platelet-associated antibody has been used to investigate thrombocytopenia in calves infected with bovine virus diarrhea virus (10) and in dogs infected with canine distemper virus (4).

Flow cytometric evaluation of platelet-associated antibody has been used by several investigators of immune-mediated thrombocytopenic purpura and HIV-1-associated thrombocytopenia (2, 21, 41, 44). Alterations in mean platelet fluorescence and the appearance of distinct populations of platelets with different levels of platelet-associated Ig have been described previously (2). The mean fluorescence index has been used previously to express data from platelet fluorescence experiments (21). This index corrects for variability between experiments by adjusting for fluorescence following incubation of platelets in buffer alone. Fluorescence indices for control horses were fairly consistent and

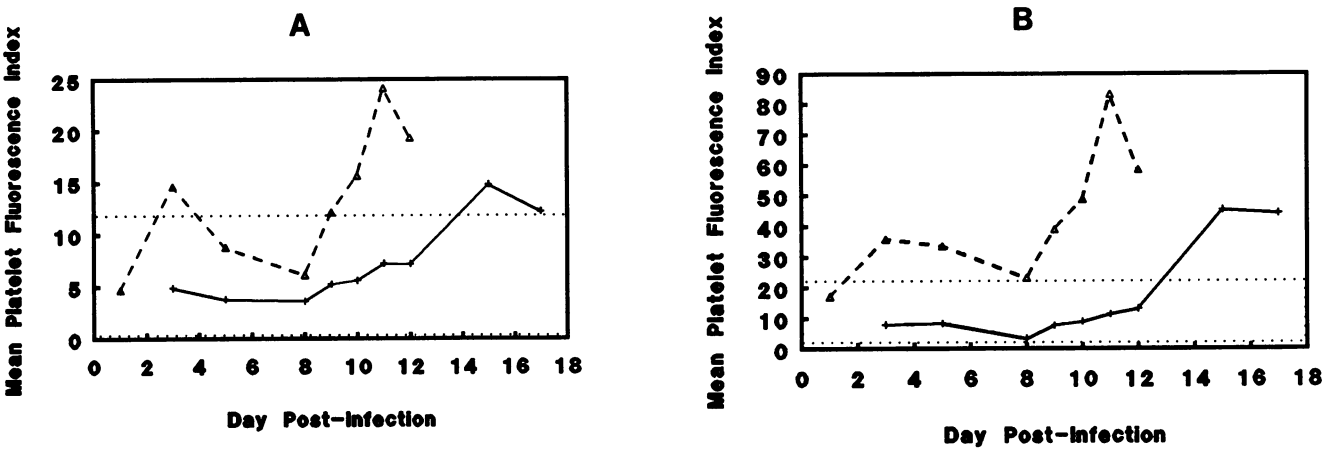

FIG. 4. Mean IgG (A) and IgM (B) fluorescence indices calculated from flow cytometric assays following infection of horses E4313 and E4316. Washed platelets were incubated for 1 h with either FITC-conjugated goat anti-equine IgG or goat anti-equine IgM, washed, and analyzed. The mean fluorescence index for each horse was calculated by dividing the mean fluorescence intensity of platelets stained with the appropriate FITC-conjugated antibody by the mean fluorescence intensity of unstained platelets. Horizontal dotted lines indicate the normal range (upper limit = mean + 2 standard deviations). The mean IgG fluorescence index for platelets from control horses was 6.05 ± 2.91, with an upper limit of normal (mean + 2 standard deviations) of 11.87. The mean IgM fluorescence index for platelets from control horses was 11.53 ± 4.87, with an upper limit of normal of 21.27. Dashed lines, indices for horse E4313; solid lines, indices for horse E4316.

within the normal limits shown in Fig. 4. Increases in platelet-associated IgG above control levels (mean + 2 standard deviations) were apparent at PID 10 in horse E4313. Platelet-associated IgM increased approximately fourfold between preinfection and PID 11 when the fluorescence index for horse E4313 peaked. This increase is comparable to the fourfold increase in platelet-associated IgM in horse E4316. Increases in both IgG and IgM in horse E4316 were seen at PID 15.

For horse E516, fluorescence indices were not calculated because of the appearance of two distinct subpopulations of platelets in that horse over the course of infection (Fig. 5). This change in platelets from some thrombocytopenic humans with immune-mediated thrombocytopenia has also been described (2). This may be due to a population of new platelets entering the circulation with either increased or decreased Ig binding, a population of activated platelets expressing more surface Ig, or a population of platelets with an altered amount of Ig because of some surface phenomenon such as capping, shedding, or spherocyte formation (2). Platelets from horse E4313 showed progressively increasing IgG and IgM fluorescence indices through PID 12. On PID 15, the fluorescence pattern changed to show two distinct populations of platelets, one of which was highly fluorescent (data not shown), very similar to the pattern shown by horse E516 (Fig. 5). It is interesting to note that the serum RT activity in these two horses (E516 and E4313) was markedly higher than the serum RT activity in the two horses (E390 and E4316) which did not develop distinct subpopulation changes in fluorescence activity.

The specificity of platelet surface antibody in horses acutely infected with EIAV was not determined in this study. However, the greater magnitude of surface IgM (Fig. 4) may indicate the presence of specific anti-EIAV antibody, since IgM synthesis following primary antigenic stimulation generally precedes IgG synthesis (33). The subjective assessment of increased platelet-associated Ig as early as PID 4 (Fig. 2) might argue against binding of virus-specific antibody. However, in experimental infection of horses with equine herpesvirus 1, increasing levels of circulating immune

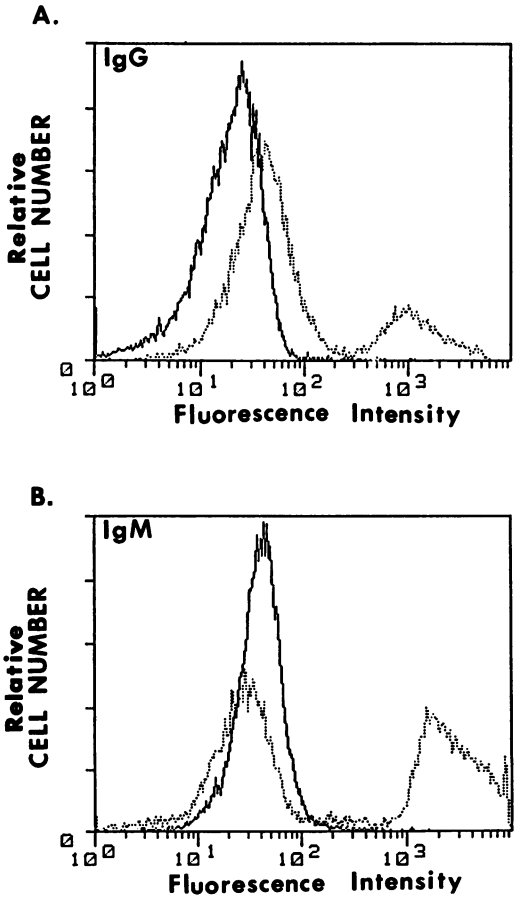

FIG. 5. Platelet fluorescence histogram of preinfection (solid line) and PID 9 (dotted line) platelets, illustrating new subpopulation of platelets showing markedly increased fluorescence, from horse E516. (A) IgG; (B) IgM.

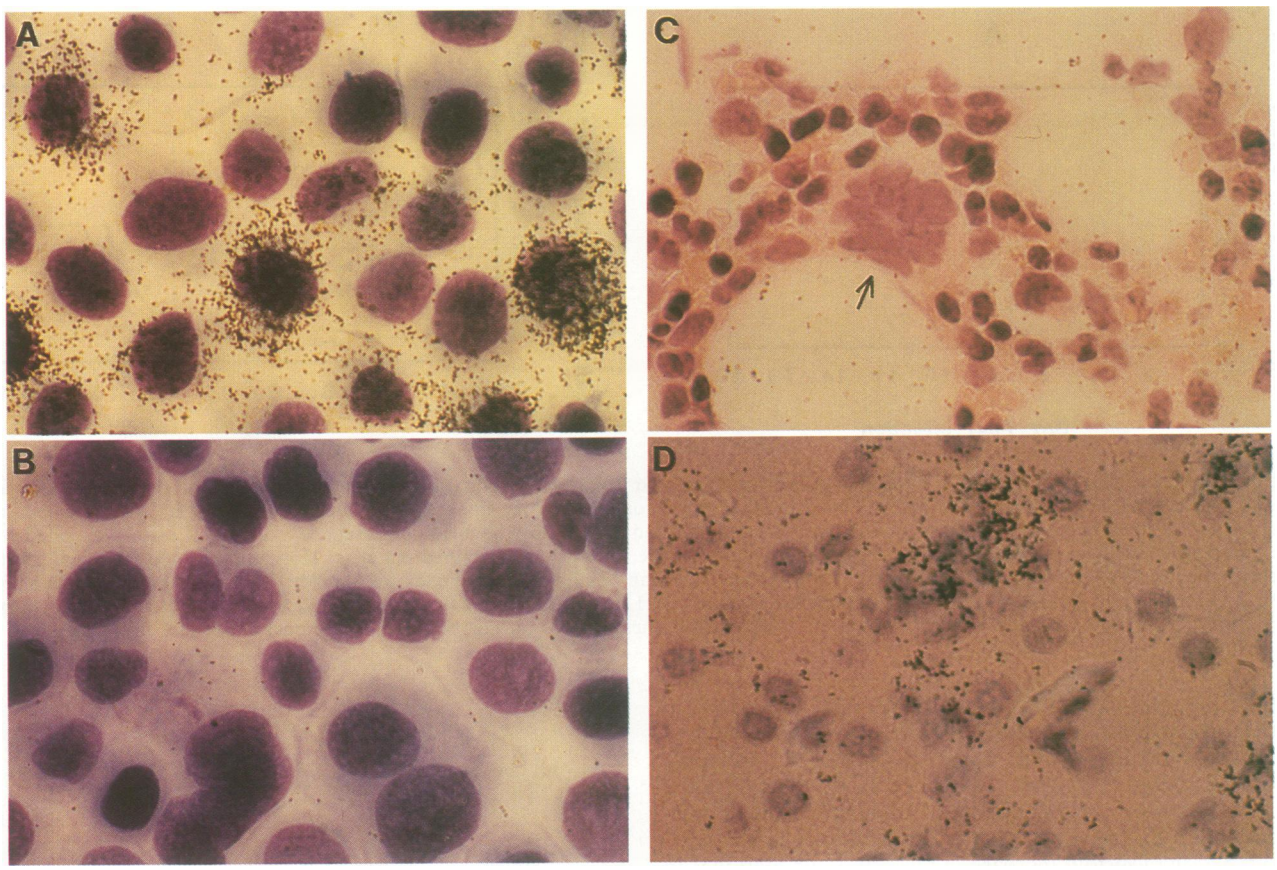

FIG. 6. In situ hybridization with EIAV-specific RNA probes. Equine dermis cells were infected in vitro with the Malmquist strain of EIAV and hybridized with the radiolabeled SP6 (A) or T7 (B) RNA probe. Bone marrow (C) and liver (D) sections from horses with EIAV, hybridized with the SP6 RNA probe, at PID 10 are shown. A megakaryocyte is indicated by the arrow in panel C.

complexes have been documented as early as PID 2 (11). If virus-specific antibody is produced in very small quantities as early as PID 2 or 3, it might initially bind to small quantities of circulating virus, and these immune complexes attach to circulating platelets. Only after platelet-binding capacity was exceeded would free serum antibody be detectable. Studies of the time course of virus-specific immune complexes in the sera of acutely infected horses are planned.

The inability to detect specific viral antigen or nucleic acid on platelets would seem to argue against virus-antibody immune complex deposition there. However, the techniques used here may not have been sensitive enough to detect relatively small quantities of platelet-bound virus. Failure to detect viral RNA with in situ hybridization may be due to disruption of immune complex binding during the processing of slides. If viral epitopes were already saturated with endogenous antibody, fluorescent-antibody detection might be prohibited. In thrombocytopenic calves infected with bovine viral diarrhea virus, indirect fluorescent-antibody testing of washed platelets demonstrated platelet-associated viral antigen in only two of seven calves, despite the observation that washed platelets from all calves were positive for virus by using an in vitro infectivity assay (10). Similarly, studies of platelets from thrombocytopenic humans infected with HIV-1 failed to detect HIV-1 antigen by enzyme-linked immunosorbent assay (17), despite the observation that washed platelets are infective in vitro (47). We plan to determine if antibody-positive platelets from EIAV-infected horses are infectious.

If viral antigen is not directly involved in immune complex deposition on platelets, an alternative explanation for increased platelet-associated Ig would be a cross-reaction of virus-specific antibody with an epitope on normal equine platelets. This would result in a persistent thrombocytopenia in chronically infected animals that have a high serum antibody titer against EIAV. No reports of a persistent thrombocytopenia in EIAV-infected horses have been described. We tested one asymptomatic, antibody-positive horse by both direct fluorescent-antibody and flow cytometric analyses and failed to detect any increase in platelet-bound Ig (data not shown).

Subjective analysis of bone marrow samples taken at necropsy suggests no alteration of numbers of megakaryocytes in any horse, even at the nadir of platelet counts. Quantitation of marrow megakaryocytes pre- and postinfection was not attempted in these horses because sacrifice was planned prior to the time of expected maximal bone marrow response and because of a difficulty in accurately assessing megakaryocyte numbers in equine bone marrow aspirates. Sequential core biopsies of bone marrow would be necessary to effectively quantitate megakaryocytes. A normal marrow response is suggested by the rapid rebound in platelet counts observed concomitant with resolution of fever in the horse allowed to cycle through multiple viremic episodes (Fig. 1).

In situ hybridization clearly demonstrates that active infection and replication of EIAV do not occur in megakaryocytes of acutely infected horses (Fig. 6). We cannot definitively rule out a defect in release of mature platelets from the marrow during acute viremia. Serial core bone marrow biopsies taken from a single horse experiencing multiple cycles of viremia and thrombocytopenia would be necessary to determine the extent of responsiveness of megakaryocytes in acute EIAV infection.

The rapid rebound in platelet counts observed with declining temperature may also indicate sequestration of platelets coated with antibody. The marked splenomegaly seen in infected horses is consistent with this theory. The reticuloendothelial cells of the spleen may simply remove damaged areas of platelet membrane and rerelease the platelets into general circulation as thrombocytopenia is resolved. We plan to investigate whether antibody-coated platelets are destroyed, or merely temporarily sequestered, by electron microscopic studies of the liver and spleen.

The splenomegaly and increased platelet-associated Ig demonstrated in these horses acutely infected with EIAV are similar to findings in thrombocytopenic humans infected with HIV-1 (1, 6, 16, 17, 20). Platelets from HIV-1-infected thrombocytopenic individuals have increased surface IgG, C3C4, and IgM, and polyethylene glycol-precipitable serum immune complex levels are three- to sevenfold greater than normal (36, 45). This led researchers to hypothesize the deposition of circulating immune complexes on platelets. But despite finding anti-HIV-1 antibody complexes on the surface of platelets from these patients, HIV antigen could not be detected by either antigen capture assay or proviral DNA gene amplification (17). Although the lack of a nucleus or host DNA in platelets would not necessarily preclude synthesis of viral DNA in platelets, it is more likely that intact virions are superficially associated with platelets, without the presence of provirus. Reverse transcription of RNA extracted from platelets, followed by polymerase chain reaction, might be more sensitive for detection of immune-complexed virions than direct polymerase chain reaction. Platelet-associated HIV-1 has been detected by other researchers using an in vitro infectivity assay and polymerase chain reaction studies (47).

In addition to immune-mediated mechanisms of platelet destruction in HIV-infected patients, active infection of bone marrow megakaryocytes has been demonstrated by in situ hybridization (48). This infection correlates with ultrastructural damage to the surface membrane of megakaryocytes and the peripheral zone of cytoplasm where blebbing and vacuolization are frequently seen (50). Internalization of HIV-1 by both megakaryocytes and platelets has been observed by electron microscopy (49). The human megakaryocytic cell line CMK is easily infected in vitro with HIV-2 and produces significant amounts of virus in culture. Infection of these cells appears to be mediated by CD4 surface antigen, and infection leads to downregulation of the receptor (34). In situ hybridization of bone marrow sections from horses in this study failed to demonstrate any replication of EIAV in megakaryocytes.

The cell types primarily responsible for removal of antibody-coated platelets from circulation (hepatic Kupffer cells and splenic macrophages) (4, 28) are also the cell types that show the highest level of viral replication during acute infection with EIAV (9). EIAV immune complexes are infectious in vitro (22), so it is possible that platelets act as virus carriers, disseminating the virus in the form of infectious immune complexes to permissive macrophage-type cells. Although this is unlikely to be the sole or primary mode of entry of EIAV into permissive cells, it may be an efficient secondary mode of penetration. Ingestion of virus by phagocytosis does not necessarily imply destruction in the phagolysosome. HIV-1 can be internalized into epithelial cells via phagocytic endosomes and cause dissolution of the phagolysosome membrane before the virus is damaged (7). Infection with HIV-1 is enhanced in vitro by the presence of specific antibody, via an F_c receptor-mediated mechanism (43).

Each of the four horses studied showed a decrease in erythrocyte numbers that roughly paralleled the decline in platelet count (data not shown). The anemia of equine infectious anemia results from both an immune-mediated clearance of erythrocytes (23, 25, 31, 37–39) and a suppression of the bone marrow regenerative response (24, 32). Erythrophagocytosis in the spleens and bone marrow of horses that were necropsied is consistent with an immune-mediated extravascular hemolysis. Since complement- and antibody-coated erythrocytes are removed from the peripheral circulation by the same cell types that remove damaged platelets, erythrocytes may also serve as inadvertent carriers of virus, targeting virions to permissive cell populations. This would also contribute to the splenomegaly and hepatomegaly seen at necropsy. In this study, the role of complement on the platelet surface was not examined. However, it is likely that complement is involved in platelet immune complexes, since it plays such an important role in erythrocyte clearance (23, 31, 38). Phagocytosis of antibody- and complement-coated platelets and erythrocytes by tissue macrophages may stimulate release of proinflammatory cytokines that induce fever. It is also possible that viral replication in the same cells potentiates cytokine release, so that cytokine release and temperature parallel viral replication. These theories remain to be tested.

Horses acutely infected with wild-type EIAV have elevated levels of platelet-bound IgM and IgG, as detected by both direct immunofluorescence assay and flow cytometric analysis. In situ hybridization of bone marrow samples revealed that megakaryocytes are not actively infected with EIAV during acute disease. These findings are consistent with an immune-mediated mechanism of thrombocytopenia, most likely because of immune complex deposition on platelets. Although some megakaryocyte suppression cannot be ruled out on the basis of these findings, the rapid rebound in platelet numbers following resolution of viremia suggests that if suppression is present, it is transient. It is possible that immune complex deposition on platelets targets viral dissemination to hepatic Kupffer cells and splenic macrophages. EIAV-induced thrombocytopenia may be a valuable model for study of lentivirus-associated thrombocytopenia. The EIAV system is particularly intriguing because of the multiple concomitant cycles of fever, viremia, and thrombocytopenia in animals infected with wild-type virus.

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